TRYPANOSOMA BRUCEI AS A FILTERABLE VIRUS—A PRELIMINARY NOTE

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The satisfactory study of a filterable virus presents many technical difficulties. The danger of drawing sweeping conclusions from improperly controlled experimental conditions is often more real than at first may be apparent. The lack of uniformity in the procedure of various investigators, together with the difficulty of establishing a type experiment have been pointed out and discussed by Meyer (1914). Much of the confusion exhibited in the results of various workers is directly attributable to the failure of duplicating closely the experimental conditions involved. Recognition of this lack of uniformity in filtration experiments and a plea for the standardization of methods was made early by Marchoux (1908). Such standardization of apparatus and technic is absolutely essential if reported findings are to be of any comparative value. The same writer calls attention to the various physical factors affecting both the filter and the material to be filtered, any one or all of which may operate to modify the results greatly. Because of these limitations, and because of the fact that standardization has not yet progressed to that point wherein results may be strictly interpreted on a comparative basis, repetition of most if not all of the earlier work using a uniform technique would seem to be highly desirable. The controversies between Remlinger, Celli and Dublasi and others concerning the virus of rabies; or of Nicolle and Adil-Bey and Todd on the virus of cattle plague, may be cited as cases in point. The type of bougie, filtration temperature and pressure, duration of filtration, dilution of the material to be filtered, albumen content of such material, as well as the nature of the diluent itself, are some of the most important factors upon which much depends.

The conception that all filterable viruses are of an invisible or 'utramicroscopic' nature has long since been abandoned. The observations of Borrel (1908) and Bosc (1902) on sheep-pox; Novy's claim regarding the filtration of *T. lewisi* (1904), Wolbach and Binger's (1914) work on *Spirochaeta elusa;* Novy and Knapp's (1906) studies on *Spirillum obermeieri;* as well as the well-known works of Guarnieri, Calkins and others on the peculiar inclusion bodies found in vaccinia, trachoma and hydrophobia, represent some of the attempts to demonstrate microscopically the filterable bodies. In those cases which have not yet yielded positive results, a further acquaintance with the life

cycle involved may possibly enable us to demonstrate a visible entity during certain stages in the development of many of the so-called 'ultramicroscopic viruses.' Of interest in this connection is the recent work of Dios and Oyarzabal on the trypanosomes of surra and of mal de caderas. These authors describe an intracorpuscular form of the specific trypanosome concerned in the blood of horses experimentally infected. Kraus, Dios and Oyarzabal claim also that there exists an invisible stage in the life cycle of certain of the bovine piroplasmas and trypanosomes. They offer these observations in support of Schaudinn's generalization that there is an invisible stage in the life cycle of some protozoa. In a recent publication Kleine criticizes these findings and attributes the "invisible stage" to a lack of careful search of the infectious material. He asserts that with proper technique and diligence the parasites may be demonstrated. Schepilewsky's claim to have seen thread-like appendages on trypanosomes by the use of dark-field illumination is also worthy of note.

With these sketchy notes pertinent to some of the problems involved, the various channels of error, and the somewhat chaotic state of our knowledge of the filterable viruses as a group, a few words may now be said relative to observations which deal more directly with the nature of the present investigation. Novy and Knapp (1906) observed that *T. lewisi* in culture underwent various morphological changes. The minuteness of some of these forms led them to experiment with filtrates of such culture material but exact details of their procedure are not available. The material was diluted with salt solution and filtered through Berkefeld candles at a pressure of five pounds. A perfectly clear filtrate was obtained which upon injection into white rats yielded three positive infections in nine attempts. Similar experiments with cultures of *T. brucei* and with suspensions of the blood and organs of infected animals yielded negative results. Wolbach, Chapman and Stevens (1915) in discussing this work state that "these experiments were done under high pressure (50 lbs. plus) with Berkefeld filters which had been reduced by sandpapering." Reference to the article by Novy and Knapp quoted above conveys the impression that such conditions obtained in the experiments with *Spirillum obermeieri* but not in the work with trypanosomes. In a personal communication, Dr. Novy refers to the importance of using cold liquids and of speed both in filtration and injection of filtrates, but mentions nothing regarding the other details. Bruce and Bateman (1908) working with both cultures and organ suspensions of animals infected with *T. brucei* or *T. evansi* were unsuccessful in their attempts to produce infection with the filtrates. They passed the infectious material through Berkefeld filters controlled with *Micrococcus melitensis* and concluded that neither trypanosome produces, in the bodies of animals or in culture, forms

that can pass through the pores of a bacteria-proof filter. Bruce and his associates (1911) working with filtrates of the intestinal tract of flies known to be infected with *T. gambiense* obtained entirely negative results. Wolbach, Chapman, and Stevens (1915) stimulated by the results of Novy and MacNeal's work with *Spirochaeta duttoni* and the confirmation of these results by Todd and Wolbach (1914), repeated their earlier experiments, using *T. brucei, T. lewisi* and *T. gambiense.* The filtration was accomplished with Berkefeld "V" filters, using pressures ranging from gravity to 50 pounds, and the results were controlled with a suspension of *B. prodigiosus* or of *Staphylococcus citreus.* Twenty-four experiments were performed, and in the case of every bacteriologically sterile filtrate, inoculation into white rats failed to infect. The control rats which had been inoculated with the unfiltered material developed infection. These writers attribute the positive results of Novy and MacNeal to the thinning down of the filters used by them and do not believe that these shaved filters would have yielded bacteria-free filtrates under the conditions of their own experiments. They state further that even in actively growing cultures they have not seen forms whose least dimension did not exceed the diameters of the bacterium used for control. In view of their results, they conclude therefore that trypanosomes from cultures and from animal tissues are not filterable through bacteria-proof filters.

Experimental Work

On January 27, 1921, through the courtesy of Dr. F. G. Novy of Ann Arbor, we received two cultures each of *T. lewisi* and *T. brucei.* These cultures were on rat's blood agar. Of six white rats injected intraperitoneally with saline suspensions of the cultures of the latter, one developed infection. From this source, a series of white rats and guinea-pigs have been inoculated and the strain maintained for study. It may be of interest to remark that dark field examination of the original culture material failed to reveal any living forms, and doubt was entertained regarding the viability of the material.

In the course of the examination of fresh and stained material derived from the blood and organs of animals infected with *T. brucei,* preparations were secured revealing curious transformations of the parasites (1921). Granular nuclear detritus; minute ovoid or round Leishmania-like forms; cycle-shaped Herpetomonad and Crithidial bodies (both flagellated and non-flagellated); and typical trypanosomes with all of the characteristics of the genus—these and other appearances suggestive of intermediate stages were often found not only in the same preparation but often in the same field. It is the writers' opinion that these bodies are not to be confused with the degeneration changes

described by Laveran and Mesnil as occurring when trypanosomes are allowed to come into contact with serum, saline and other deleterious substances. They may or may not be related to the 'latent bodies' of Moore and Breinl or to the schizogenous forms described by Walker in the spleen of animals infected with *T. evansi.* It was at first thought that these findings were exceptional. It has since been found possible to demonstrate them on numerous occasions in the liver, spleen and occasionally the heart's blood of animals at or shortly after death. A search of the organs before death had supervened has not yet been undertaken. It is reasonable to presume however that such morphological changes from supposed type will be encountered to some degree at least at some appreciable time before the death of the animal.

The routine procedure in the filtration experiments has been as follows: The material used came from animals handled at or as closely after death as possible. The animal was secured on a small dissecting board and the thoracic and abdominal viscera were exposed with aseptic precautions. This care was exercised since contaminating organisms naturally would confuse the interpretation of the bacterial control. The heart was removed in toto and placed immediately in a sterile mortar containing 30 to 40 cc. of sterile citrated saline (10% sodium citrate in physiological saline). The blood in the thoracic cavity was aspirated with a sterile Luer syringe without needle. Portions of the lungs, liver, spleen, kidneys, inguinal lymph nodes and bone marrow were also removed and placed immediately in the same saline. In each case, smears of the various organs were made and later stained by the writers' modification of Wright's method (1921). The material in the saline was then minced carefully with a pair of sterile scissors and then ground further with a sterile pestle. To the mixture was then added a loopful of a 24-48 hour slant culture of *B. prodigiosus.* After mixing, the maceration was filtered through one or two thicknesses of sterile gauze directly into the aluminum receptacle enclosing the filter candle. This procedure was carried out immediately after death with at least half of the series. In one successful instance however, approximately 36 hours had elapsed before autopsy.

With Mandler filters of diatomaceous earth, the filtration was accomplished by means of a suction pump using pressures varying between twenty and twenty-five pounds and with time from twenty-five to forty-five minutes in different experiments. Preliminary examination had been made of all animals in order to be assured that no trypanosomes of any sort were present before using for experimental purposes. Moreover these animals had been in stock for many weeks before use. The filtration usually yielded approximately 3 cc. of colorless or straw-tinted liquid. One or two guinea-pigs were inoculated intraperitoneally with 1.5-2 cc. of the filtrate. A control animal was inoculated with the

same quantity of the unfiltered material. Immediately following this process, three 2 mm. loopfuls of the filtrate were placed on glucose agar to determine the absence of *B. prodigiosus* while at the same time one loopful of the original maceration was placed on another slant. The purpose of this technique was of course to furnish proof that the candle was of grain sufficiently fine to intercept the bacterial micro-organism. Such tubes were then incubated at 37 C. for 24 hours, followed by room temperature for one week to insure maximum production of pigment.

To date twenty-five experiments have been performed. In four of this series, the filters proved defective by bacteriological control. In two other cases, the control animals failed to develop infection. In the nineteen remaining experiments wherein the control animals developed positively and in which bacteriological cultures of the filtrate remained negative for growth, ten of the animals inoculated with bacteria-free filtrates developed infection of *T. brucei.*

Discussion and Results

The possibility of the existence of forms still smaller than those described in the stained smears, and their relation or identity with the filter-passing entity, must be borne in mind as a distinct possibility. The divergence in the results reported by various investigators may be due to (1) differences in the technique, (2) peculiarity of the particular strain, and (3) to the presence or absence of the specific form changes alluded to. In the opinion of the writers, the last condition should prove to be an important postulate.

1. Form changes are described in the blood and organs of white rats and guinea-pigs dying of infection with *Trypanosoma brucei.*

2. The viability of these forms is evidenced cytologically by the mitotic figures and physiologically by their infectiveness.

3. The blood and organs of guinea-pigs dying or dead of infection with *T. brucei,* and in which the form changes described occur, when filtered through a bacteria-proof filter, yield filtrates which in ten cases out of nineteen were infective for guinea-pigs.

4. The possible relation between this filterable virus and certain of the described 'involution forms' or with a problematical ultramicroscopic stage in the life of this parasite is pointed out.

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